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THE EFFECTS OF PYRIDOSTIGMINE AND PHYSOSTIGMINE
ON THE CHOLINERGIC SYNAPSE

ANNUAL REPORT

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Neuromuscular junctions (NMJs) from diaphragm, soleus and extensor digitorum longus muscles of male albino rats were assessed for morphological alterations following acute exposure (30 minutes) by single subcutaneous injections of pyridostigmine bromide (0.36 to 1.0 mg/kg) and following subacute exposure (2-14 days) by subcutaneously implanted osmotic minipumps that contained 10 mg/ml of pyridostigmine. Recovery processes were monitored up to 60 days. Drug exposure resulted in pre- and postsynaptic alterations. These included partial withdrawal of the nerve terminals from junctional folds, disruption of the myofibrillar organization and damage to membrane-bound organelles. These lesions were observed as independent occurrences in some NMJs but were present concurrently in other fibers. The extent of the pathology was dose-dependent. Analysis of the data allowed three conclusions to be formulated: (1) Animals with a whole blood					
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cholinesterase (ChE) depression of 60-70% exhibited alterations in all three muscle fiber types. These lesions appeared similar in severity, indicating that pyridostigmine does not selectively affect one muscle fiber type at this level of ChE depression. (2) The alterations appeared similar in nature and extent following drug exposure by single injection and by osmotic minipump. Thus at 60-70% ChE depression, the method of drug administration did not affect the severity of the pathology. (3) At least some NMJs of all three muscles underwent a period of partial denervation following acute (ChE depression approx. 90%) or subacute (ChE depression approx. 70%) pyridostigmine exposure. However, recovery was in process and sometimes complete 60 days following drug exposure, indicating that pyridostigmine-induced alterations are reversible.

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SUMMARY

The quaternary carbamate, pyridostigmine bromide, has been suggested for use in prophylaxis against intoxication with irreversible cholinesterase inhibitors. Since virtually no anatomical data were available concerning the neuromuscular toxicity of the drug, a study was undertaken to evaluate the effects of acute and subacute doses of pyridostigmine on the ultrastructure of rat diaphragm neuromuscular junctions (NMJs) and muscle fibers. The results of these investigations were reported in the annual report for Year 1 (April 1, 1983 - March 31, 1984) of contract DAMD-17-83-C-3126. The data suggested a dose-dependent effect of pyridostigmine which resulted in both pre- and postsynaptic alterations of the diaphragm NMJs. During Year 2 of the contract, the investigations have been expanded to include analyses of the effects of different modes of pyridostigmine administration on three muscles (diaphragm, extensor digitorum longus and soleus). In addition, physiological recovery processes, as reflected by morphological changes, have been assessed during extended periods following drug exposure. The results of these studies are summarized below and detailed in the Methods, Results, and Discussion sections of this report.

Neuromuscular junctions from diaphragm, soleus and extensor digitorum longus (EDL) muscles of male albino rats were assessed for morphological alterations following acute (30 Minute) and subacute (2-14 day) exposure to pyridostigmine and during postexposure recovery periods of up to 60 days. The experiments were designed to provide data necessary to accomplish three goals which were: (Goal 1) to compare the effects of drug administration by single injection and by osmotic minipump (continuous infusion), (Goal 2) to determine if pyridostigmine selectively affects fast or slow twitch muscle fibers, and (Goal 3) to monitor and evaluate morphological changes during long-term recovery.

To complete Goals 1 and 2, the diaphragm, soleus and EDL muscles were selected to compare the effects of the method of drug administration (injection versus infusion) on muscles of different fiber type composition. The diaphragm has approximately equal numbers of type I and type II fibers while the soleus and EDL possess primarily type I and type II fibers respectively. Pyridostigmine was administered to each acute exposure animal by a single subcutaneous injection of 0.36 mg/kg pyridostigmine and to each subacute exposure animal by a subcutaneously implanted osmotic minipump containing 10 mg/ml pyridostigmine. Both treatments resulted in a whole blood cholinesterase (ChE) depression of approximately 60-70% as determined by radiometric assay. Control animals received only Mestinon^R-equivalent diluent.

Both acute and subacute exposures resulted in morphological alteration of the NMJs of all three muscles, although considerable variation in the extent of damage occurred even within individual NMJs. The most frequently observed presynaptic alterations were mitochondrial damage and partial withdrawal of nerve terminal branches (partial denervation). Postsynaptic changes included occasional rarefaction of mitochondrial matrices and disruption of the myofibrillar organization in small numbers of subjunctional sarcomeres. The data indicate that acute or subacute exposure to pyridostigmine bromide at a whole blood ChE depression of 60-70% results in similar alterations to the NMJs of three muscles with substantially different fiber type populations. At this dose level, the severity of the damage varied from fiber-to-fiber in an apparently random manner and did not appear to be related to a specific fiber type or dosage regimen.

To accomplish Goal 3, long term recovery processes were evaluated following exposure to 1.0 mg/kg pyridostigmine by single injection and following 14 days of exposure to pyridostigmine by osmotic minipump (total dose 20 mg pyridostigmine). Recovery was analyzed at 7, 14, 21, 35 and 60 days following withdrawal from drug. Whole blood cholinesterase activity levels were monitored by radiometric assay before, during and periodically following drug exposure. Following a 30 minute exposure to 1.0 mg/kg, ChE levels were depressed to approximately 90% of preinjection values. Animals exposed to 10 mg/ml pyridostigmine maintained a relatively constant ChE depression of approximately 70% throughout the 14 day exposure period. Preliminary data indicate that postexposure ChE values initially exceeded pre-exposure ChE levels and then fluctuated at least through day 35 of recovery.

At the dose levels studied, NMJs of all three muscles initially underwent the typical pre- and postsynaptic alterations described above. As previously reported, signs of partial denervation increased during the 35 day period following pyridostigmine exposure by single injection or minipump. However, indications of terminal sprouting were also apparent by day 21 of the recovery period. Although some pre- and postsynaptic morphological irregularities persisted at day 60 of recovery, the general trend appeared to be toward recovery of pre-exposure morphology in all three muscles. At present, it appears that the soleus may undergo a longer recovery period than the diaphragm and EDL.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

Summary.....	2
Foreword.....	4
Table of Contents.....	5
Statement of Problem.....	6
Background.....	8
Materials and Methods.....	9
Results.....	11
Discussion.....	16
Recommendations.....	18
Literature Cited.....	19

STATEMENT OF PROBLEM

Pyridostigmine bromide, a reversible anticholinesterase drug, has been suggested for use in prophylaxis against intoxication with irreversible cholinesterase inhibitors. Recent investigations have indicated that *in vivo* exposure to the drug results in morphological alterations to the NMJs of the rat diaphragm muscle (Hudson and Foster, 1984; Hudson, et al., 1985). In these studies, acute doses of pyridostigmine bromide in Mestinon[®]-equivalent buffer were administered by single subcutaneous injection in doses that ranged from 0.0036 to 3.6 mg/kg (0.001 - 1.0 LD50). Tissues were analyzed in the acute experiments 10 - 30 minutes and 2 and 7 days postinjection. Subacute exposures were administered by subcutaneously implanted osmotic minipumps that contained either 3.0 or 20 mg pyridostigmine in Mestinon[®]-equivalent buffer. Tissues were analyzed after subacute exposures of 3, 7 or 14 days and with post-exposure recovery times of 7, 14 or 23 days. In select animals, whole blood cholinesterase activity was measured by radiometric assay. The morphological effects of the drug were localized predominantly at the neuromuscular junction with both pre- and postsynaptic regions involved. The data indicated that there was variation in the extent of the damage between different muscle fibers and between different areas of an individual NMJ. With acute doses, there was a dose dependent response which was manifest in terms of the location of the pathology. Low doses seemed to affect the presynaptic area with no apparent effects on the muscle cell. At higher doses (0.01 - 1.0 LD50), both pre- and postsynaptic elements became involved. The subacute doses examined also created dose dependent lesions in the diaphragm. At 14 days, the 20 mg dose group exhibited pre- and postsynaptic alterations which were more extensive than those seen in the 3 mg group although no subacute dose created damage equivalent to that seen 30 minutes after a single LD50 dose. Presynaptic alterations included withdrawal of the terminal from the junctional folds, invasion of the synaptic cleft with Schwann cell processes, and disruption of axon terminal organelles. postsynaptic alterations included subjunctional supercontraction, disruption of myofibrillar apparatus with the z-lines apparently being the most sensitive element, and disruption of subjunctional mitochondria. Ultrastructural recovery of the diaphragm after acute or subacute exposure to the drug varied. The evidence suggested that subacute exposure had the greatest effect within the first week of exposure with additional exposure sustaining but not appreciably increasing the extent of the lesion. Diaphragms subjected to moderate acute doses exhibited a progression in the degenerative process (including partial denervation) for at least one week post-exposure. Evidence of recovery from the drug-induced effects included reinnervation of junctional

areas and the replacement or repair of subcellular elements. In summary, data collected in Year 1 indicated a dose-dependent effect of pyridostigmine on muscle ultrastructure with evidence of subsequent recovery.

While the data gathered during Year 1 added significantly to the knowledge concerning the ultrastructural effects of pyridostigmine, the findings also prompted a number of additional questions. In answer to some of these, the experiments conducted in Year 2 were designed to accomplish the following:

- a. to subjectively establish if pyridostigmine exposure by single injection induced more severe or extensive damage than exposure by osmotic minipump when both methods resulted in similar ChE depressions.

- b. to determine if pyridostigmine selectively damages one fiber type more severely than another.

- c. to assess the recovery processes in pyridostigmine-exposed diaphragm, EDL and soleus NMJs to determine if the three muscles recover in a similar manner and time frame.

BACKGROUND

Pyridostigmine is an anticholinesterase drug capable of rapidly inhibiting acetylcholinesterase in mammals by covalent enzyme carbamylation. Clinically in its Mestinon formulation, the drug is used primarily in the treatment of myasthenia gravis. Experimentally when combined with cholinolytic and oxime therapy, pyridostigmine has been shown to be an effective prophylactic against systemic exposure to irreversible cholinesterase inhibitors. Pyridostigmine prophylaxis is due, presumably, to the continuing production of a non-inhibited pool of acetylcholinesterase which is derived from the spontaneous decarbamylation of the previously pyridostigmine-inhibited enzyme (Barry and Davie, 1970; Gordon, et al., 1978; Dirnhuber, et al., 1979).

Numerous reports have established that the irreversible cholinesterase inhibitors produce myopathies and/or neuropathies and are responsible for abnormal physiology at the mammalian neuromuscular junction (Preussner, 1967; Ariens, et al., 1969; Fenichel, et al., 1972, 1974; Laskowski, et al., 1975; Wecker and Dettbarn, 1976; Wecker, et al., 1979; Salpeter, et al., 1979, 1982). Until recently, the quaternary carbamate neostigmine was the only carbamate anticholinesterase drug employed in anatomical studies to evaluate the potential toxicity of this class of drugs vis-a-vis neuromuscular pathology (Engel, et al., 1973; Engel and Santa, 1973; Ward, et al., 1975; Hudson, et al., 1978). The paucity of anatomical data available concerning pyridostigmine-induced neuromuscular alterations prompted our recent ultrastructural study which assessed presynaptic damage resulting from pyridostigmine exposures (Hudson, et al., 1985). The data revealed that acute or subacute administration of pyridostigmine resulted in withdrawal of localized portions of the nerve terminal from the junctional folds, invasion of the synaptic cleft by Schwann cell processes and disruption of nerve terminal organelles (Hudson, et al., 1985). Postsynaptic changes included disruption of myofibrillar organization and damage to membrane-bound organelles (Hudson, unpublished data). The severity of both pre- and postsynaptic alterations was dose dependent.

These results indicated the necessity to extend the investigations to establish additional information concerning the ultrastructural effects of pyridostigmine. Thus, experiments were designed to determine if drug administration by single injection induced similar or more severe NMJ pathology than drug administration by continuous infusion (osmotic minipump) when comparable ChE depressions were maintained. Secondly, the NMJ alterations were compared in the diaphragm, EDL and soleus to establish if pyridostigmine

selectively affects one fiber type more severely than another. Finally, NMJs of all three muscles were monitored to confirm Year 1 data that indicated recovery processes occurred following pyridostigmine exposure.

MATERIALS AND METHODS

Male albino rats (Edgewood or Charles River strains) weighing 180 to 250 gms received subcutaneous, acute and subacute exposures to pyridostigmine bromide. All acute exposures were by single syringe injections under the skin of the midback region and all subacute exposures (maximum 14 days) were via osmotic minipump (Alzet^R 2ML2 minipump; ALZA Corp., Palo Alto, Ca.) implanted under the skin of the midback. Pyridostigmine bromide was administered in a Mestison^R-equivalent diluent composed of 1.30 mg/ml citric acid monohydrate, 4.10 mg/ml sodium citrate dihydrate, 0.50 mg/ml methyl paraben, 0.05 mg/ml propyl paraben and 7.40 mg/ml sodium chloride in sterile water at pH 5.1.

Acute Drug Exposure. Acute doses of pyridostigmine ranged from 0.0036 mg/kg to 3.6 mg/kg (1LD)subcutaneous injection = 3.6 mg/kg determined by probit analysis). Animals in the acute control group received a single injection of the Mestison^R-equivalent diluent. A minimum of 3 experimental and 2 control animals were prepared for each dose level analyzed. These animals were sacrificed under deep barbiturate anesthesia by tranacardiac vascular perfusion 10 - 30 minutes, and 2, 7, 21, 35, 60, and 90 days postinjection. These time periods allowed evaluation of acute drug effects as well as short and long term recovery processes.

Subacute Drug Exposure. Subacutely treated animals had 2 ml osmotic minipumps implanted subcutaneously to provide continuous infusion of pyridostigmine. Use of osmotic minipumps allowed continuous release of the drug in order to maintain both tissue-ChE and tissue-drug levels as constant as possible.

Subacute doses were studied using minipumps loaded with 10.0 mg/ml pyridostigmine bromide in the Mestison^R-equivalent buffer. Control animals were implanted with minipumps containing the Mestison^R-equivalent buffer. The morphological effects of chronic infusion of pyridostigmine were evaluated after periods of 2, 7 and 14 days exposure. The processes of recovery from subacute pyridostigmine exposure were assessed 7, 14, 21, 35, and 60 days after a 14 day exposure by minipump. A minimum of 3 experimental and 2 control animals were prepared and analyzed for each dose at each exposure period. Since the Alzet^R pumps utilized in

this study released their contents at approximately 5.9 μ l/hr, the experimental animals were exposed to an approximate total of 2.8, 10 and 20 mg of drug on 2, 7 and 14 days, respectively. Blood ChE levels of each drug-treated and control animal were analyzed (Siakotos et al., 1969 see below) periodically (at least twice) during the course of the experiment.

Preparation for Electron Microscopy. All animals were prepared by whole body perfusion through the left ventricle using an initial perfusate of KCL, 5.0; $MgCl_2$, 1.0; $CaCl_2$, 2.0; $NaHCO_3$, 15.0; and Na_2HPO_4 , 1.0 containing 10 units/ml of heparin followed by the fixation perfusate containing 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4). The perfusates were maintained at room temperature. The diaphragm and both soleus and EDL muscles of each animal were immediately removed and fixed for an additional hour in cold 2.5% glutaraldehyde. NMJs were identified by staining for ChE in a solution of 5 mg acetylthiocholine iodide, 6.5 ml 0.1 M sodium cacodylate buffer (pH 7.4) with 0.2 M sucrose, 0.5 ml 0.1 M sodium citrate, 1.0 ml 30 mM copper sulfate and 1.0 ml ddH₂O (modified from Karnovsky and Roots, 1964). Endplate regions and tissue remote from the endplates were removed by careful dissection, postfixed in 1% OsO_4 , stained en bloc in aqueous uranyl acetate or 2% uranyl acetate in 70% ethyl alcohol embedded in an Epon-Araldite mixture (10% Polybed 812, 20% Araldite 70°C for 24 hours). Ultrathin sections were cut with a diamond knife using an LKB III or IV ultramicrotome, poststained with lead citrate (Venable and Coggeshall, 1965) and aqueous or methanolic uranyl acetate and examined with a JEOL 100 CX electron microscope.

Cholinesterase Assay. The radiometric method of Siakotos et al. (1969) using C-acetylcholine as the ChE substrate was utilized throughout. The relative ChE depression in whole blood (i.e., percent carbamylation of the enzyme) produced by acute or subacute exposure to pyridostigmine was determined. Blood (100 μ l) was drawn from the tail into heparinized capillary tubes and processed for an immediate assay or frozen in liquid nitrogen for subsequent assay. In order to test for the effects of freezing whole blood on the assay results, the 100 μ l blood was divided into two 50 μ l aliquots, one used for immediate assay and the other frozen for later assay. Freezing prior to assay resulted in a variation of approximately 5% of the ChE depression established by immediate assay. This was within an acceptable range for the purpose of the present study.

The enzyme assay was performed in the following manner. Up to 100 μ l (usually 5 - 50 μ l) of blood sample was added to 100 μ l 0.1M sodium phosphate (pH 7.4), 10 μ l $MgCl_2$ and 100 μ l ¹⁴C-acetylcholine. The solution was mixed immediately and

incubated for 5 minutes at 37 °C (with shaking). The reaction was stopped by adding sufficient resin-dioxane mixture (20g Amberlite CG - 120 x 8 and 100 ml dioxane) to increase the total volume to 5 ml. The enzymeresin-dioxane volume was then brought to 10 ml with dioxane, mixed by inversion (3 times) and centrifuged at 900g for 3 minutes. Then 1.0 ml of supernatant was combined with 10ml of cocktail and the radioactivity assayed in a Beckman LS-7800 liquid scintillation counter. A reagent blank was run simultaneously using water in place of sample.

Since it was not practical to gather the ChE values for all of the acutely-treated animals, a parallel study (using a group of rats not utilized in the morphological study) was employed to establish some of the data for a dose-response curve of enzyme carbamylation at the time point of 30 minutes post-injection (single subcutaneous). Animals in the recovery studies were assessed for whole blood ChE levels periodically throughout the 90 day period following exposure by single injection. A minimum of three drug-treated and one control animal was used for the determination of total whole blood ChE depression at each dose.

For the animals which were in the subacute exposure groups, ChE levels were assayed before and periodically after the implantation of the osmotic minipump. The assay was performed on these animals (a) to confirm that each minipump was releasing the drug, (b) to establish the general dose/ChE depression relationship for each dose and each animal and (c) to determine the recovery of ChE levels during the 90 day period following exposure by single injection.

RESULTS

General Observation. Animals in the acute and subacute treatment groups were monitored for behavioral signs of pyridostigmine intoxication periodically during the period of drug exposure. Animals in the acute exposure groups received single subcutaneous injections ranging in dose from 0.36 to 1.0 mg/kg pyridostigmine. Control animals received single injections of diluent. Animals were assessed for outward behavior responses 30 minutes following injection. The behavior of animals in the recovery studies were also regularly evaluated throughout the postexposure period. No obvious signs of anticholinesterase intoxication were detected in any of the animals in this acute exposure study.

Similarly, animals implanted with osmotic pumps containing 20 mg (10 mg/ml) of pyridostigmine or diluent exhibited no detectable behavioral signs of pyridostigmine intoxication

over the 14 day period of exposure or during subsequent recovery periods of up to 90 days with the exception of four animals. These 4 of 50 rats exhibited increased ocular secretions (chromodacryorrhea) within the initial few hours of pyridostigmine exposure but displayed no other typical signs of anticholinesterase intoxication. The chromodacryorrhea cleared within the first 24 hours of exposure and did not reappear. These animals as well as the rest of the experimental animals remained symptom-free for the duration of the experiment.

Whole Blood Assays. Whole blood ChE levels were determined by radiometric assay immediately prior to drug exposure for both short term and recovery groups. To determine the acute drug effects, pretreatment ChE values were compared to the ChE activity 30 minutes postinjection (Graph-1). Control animals (n=7) maintained an average ChE activity of 98.1% (SD \pm 9.3). In comparison, control animals for the subacute exposure group (n=18), had an average ChE activity of 90.8% (SD \pm 8.6) when assayed two days following implanting of minipumps. The effect of pyridostigmine administered by osmotic minipump was reflected in significantly decreased ChE activities in experimental animals (Graph 2). For the purpose of comparing short term effects of pyridostigmine administered by injection versus minipump, it is important to note that drug treatment resulted in an average ChE activity of 30.3% (SD \pm 5.4, n=4) for the acute group and 38.5% (SD \pm 6.2, n=4) for the subacute group. Since the acute and subacute exposures used in this portion of study resulted in comparable reductions in ChE activity (60-70%), a comparison of the morphological alterations associated with the two types of drug administration was made.

Morphological recovery of the NMJs of diaphragm, EDL and soleus following a single injection of 1.0 mg/kg or 14 day minipump exposure is being assessed. Whole blood ChE levels have been monitored at intervals through 60 days (90 day studies are in progress), following withdrawal from pyridostigmine. While detailed analysis is not yet complete, the minipumps depress whole blood ChE levels approximately 70% (Graph 2), through the 14 day period of exposure. Following the 14 day exposure period, the ChE levels were frequently elevated above initial pre-exposure values for up to 35 days post-exposure (ChE levels of animals in longer recovery groups is currently being assayed).

Ultrastructural Observations. NMJ structure was similar for control preparations whether the animals were subcutaneously treated with Mestinon^R-equivalent diluent via single injection (Fig. 1) or by osmotic minipump (Fig. 2). Similarly, no observable difference was present between the general morphology of NMJs from acute and subacute control

In both cases, nerve terminal branches possessed numerous synaptic vesicles and several mitochondria, each with a homogeneous matrix. The nonsynaptic surfaces of the terminals were overlaid with Schwann cell processes, while the synaptic surfaces were closely opposed to the basal lamina of the primary synaptic cleft. The 50 nm cleft was limited on the postsynaptic border by the crests of the junctional folds. Each postsynaptic mitochondrion also had a homogeneous matrix. The sarcomeres were very regular in length with myofibrillar components organized into easily recognizable A and I bands and 2 lines. These features were also typical of NMJs from diaphragm and soleus muscles from acute and subacute control animals (not shown).

Early Effects of Pyridostigmine Exposure by Injection and Minipump. A 30-minute exposure to 0.36 mg/kg pyridostigmine resulted in pre- and postsynaptic alterations of the NMJs of all three muscles (Figs. 3, 4 and 5). NMJs in the diaphragm frequently displayed localized regions of nerve terminal withdrawal from the junctional folds (Fig. 3). Synaptic vesicles often appeared so densely packed that virtually no space remained to accommodate the amounts of terminal cytoplasm observed in control terminals. Some mitochondria appeared similar to those of control terminals (Fig. 3), while others possessed swollen regions like those illustrated in the NMJs of the soleus (Fig. 4) and EDL (Fig. 5). This variation of presynaptic mitochondria structure was typical in the NMJs of all three muscles.

Postsynaptic mitochondria also exhibited a range of morphologies following an acute exposure of 0.36 mg/kg of drug. As illustrated, mitochondria of the diaphragm (Fig. 3), soleus (Fig. 4) and EDL (Fig. 5) contained rarefied areas in the matrix. Occasional mitochondria of all three muscles were also associated with membranous, lamellar structures (Figs. 4 and 5). In addition, similar multilayered membrane structures (Fig. 4) and other large vesicular structures containing membrane debris (Fig. 3) were observed. These were of undetermined origin. Sarcomere structure was generally unaltered, although the subjunctional myofibrillar structure was disorganized in occasional NMJs.

Subacute exposures also resulted in morphological alterations to the diaphragm (Figs. 6 and 7), soleus (Fig. 8) and EDL (Fig. 9). These changes were similar in nature to those observed following the acute exposure. In these figures, swollen presynaptic mitochondria are more prevalent in diaphragm NMJs (Figs. 6 and 7) than in soleus (Fig. 8) and EDL (Fig. 9). These changes were similar in nature to those observed following the acute exposure. In these figures, swollen presynaptic mitochondria are more prevalent in diaphragm NMJs (Figs. 6 and 7) than in soleus (Fig. 8) and EDL (Fig. 9) NMJs. Local separation of pre- and postsynaptic components was common (Figs. 8 and 9). In some regions, the crests of several junctional folds lacked the typical opposing nerve terminal (Fig. 8). Instead Schwann cell

processes frequently occupied the entire area normally occupied by the nerve terminal. Such images were present in all muscle types examined. In other cases, the nerve terminal remained but was separated from postsynaptic components by intervening Schwann cell processes (Fig. 9).

Postsynaptic alterations were also similar to those resulting from acute exposure to pyridostigmine. Characteristic swelling or rarefaction of mitochondrial matrix was often observed (Fig. 6 and 7), but was extremely variable. Note that in other NMJs, mitochondria appeared unaltered (Fig. 8) or reflected differences in the matrix density (Fig. 9, compare mitochondria 1 and 2). In some subjunctional regions grossly altered mitochondria contained multi-layered membrane structures (Fig. 6, arrowhead; Fig. 7). While these differences have been illustrated in different NMJs from different muscles, the same type of variability was present within each muscle. Changes in sarcomere organization was also variable. Note the total loss of myofibrillar organization in some NMJs (Fig. 7) while more subtle changes were present in the z lines and myosin and actin filament organization in other NMJs (Fig. 9). Compare Figures 6 and 7 which emphasize the variability of damage observed in diaphragm NMJs. In these micrographs, the NMJs are from different animals, but similar variability was observed within a single muscle and was also present in the NMJs of soleus and EDL.

Recovery Following Pyridostigmine Exposure By Injection and Minipump. A 30 minute exposure of diaphragm (Fig. 10), soleus (Fig. 12) and EDL (Fig. 14 and 15) NMJs to 1.0 mg/kg of pyridostigmine resulted in ultrastructural changes very similar in nature to those observed after a 30 minute exposure to 0.36 mg/kg of drug. The only significant difference was a consistent tendency toward increased severity of the pathology. The most notable presynaptic alterations were evidenced as occasional aberrant mitochondria (Fig. 14,15), partial withdrawal of nerve terminal areas from postsynaptic junctional folds (Fig. 10,15) or separation of the pre- and postsynaptic components by intervention of Schwann cell processes (Fig. 12,15). Postsynaptic alterations were also similar to those observed at lower doses. Mitochondria frequently possessed rarefied or swollen regions (Figs. 10, 12, 15) and the myofibrillar organization of the sarcomeres was sometimes disrupted (Figs. 10, 15). While these various alterations appear differently in some of the micrographs of the three different muscles, it is important to note that considerable variation occurred. This variation is emphasized in Figs. 14 and 15 which reflect the difference in the extent of damage within the EDL. The damage to the NMJ in Fig. 14 appears modest in comparison to

the partial denervation, as well as, mitochondrial and myofibrillar involvement apparent in Fig. 15. Similar disparity existed in the degree of damage evidenced from one NMJ to the next in the diaphragm and soleus muscles.

Similarly, a 14 day exposure of pyridostigmine via osmotic minipump (total exposure 20 mg) reflected the same type of alterations (Figs. 16, 20) observed with shorter drug exposure. However, partial denervation was more extensive in the NMJs of all three muscles and postsynaptic mitochondrial involvement was much less pronounced (Figs. 16, 18, 20).

Recovery was observed in all three muscles following both dosage regimens (Figs. 11, 13, 17, 19, 21). The results of the acute studies are illustrated in the diaphragm (Fig. 10) and soleus (Fig. 13) and in all three muscles following subacute exposure (Figs. 17, 19, 20). While modest mitochondrial involvement sometimes persisted (Fig. 13), this type of damage was not commonly observed (Figs. 10, 17, 19, 21). The most consistently observed alteration remaining after 60 days of recovery from acute and subacute pyridostigmine exposure was Schwann cell intervention between the nerve terminal and junctional fold crests (Fig. 11, 19). In addition, postsynaptic nuclei often possessed very irregular margins and were frequently associated with numerous Golgi.

DISCUSSION

Acute and subacute doses of pyridostigmine bromide which caused whole blood ChE depression of 60-90%, resulted in pre- and postsynaptic morphological alterations to every diaphragm, soleus and EDL NMJ analyzed. Presynaptic damage included swollen mitochondria and partial withdrawal of nerve terminal branches. These two alterations were sometimes observed in the same NMJ but also occurred independently. Mitochondrial changes presumably reflect altered ionic concentrations within the presynaptic compartment. Changes in the spatial relationship of the nerve terminal and the junctional fold crests were present as Schwann cell processes in the primary cleft and/or by withdrawal of entire terminal portions. Schwann cell processes have been reported as a regular feature in the primary clefts of frog NMJs, but occur only rarely in the control NMJs of rat muscles. Regional withdrawal of a small proportion of mammalian NMJs occurs on a continuing basis in the normal process of synaptic turnover (Cotman, et al., 1981). In the case of pyridostigmine treated NMJs, Schwann cell processes in the cleft and partial withdrawal of nerve terminals act as a type of partial denervation. Both phenomena effectively reduce the amount of optimally functioning synaptic surface available. It is apparent that this decrease in NMJ functional area is enhanced by pyridostigmine exposure although the mechanism responsible for the change is not known. It should be noted that the reduction in apposing pre- and postsynaptic surfaces does not necessarily imply a concomitant reduction in neuromuscular function. Some portions of each NMJ appear morphologically sound and may possess the ability to function at a level which does not significantly impair animal behavior. Appropriate electrophysiological studies are required to assess the effect of decreased synaptic area on function.

Pyridostigmine-induced postsynaptic damage included changes in membrane bound organelles,, most notably the mitochondria, and/or disruption of sarcomere organization. These two categories of alterations sometimes occurred in the same NMJ but were not mutually dependent. Abnormalities in membrane bound organelles presumably reflects inappropriate ion concentrations resulting from drug exposure. Extremely high levels of Ca^{++} accumulate in the endplate regions (Salpeter, et al., 1982). These high levels of Ca^{++} may also result in disruption of myofibrillar integrity by mediating Ca^{++} -activated proteases which attack particular muscle proteins (Salpeter, et al., 1982). The myofibrillar changes associated with the acute and subacute doses of pyridostigmine used in this investigation, are mild in comparison to those seen at higher acute doses (Hudson, et al., 1985).

Since the diaphragm, soleus and EDL have different fiber type compositions (Hudson, et al., 1982) the possibility existed that exposure to pyridostigmine might affect the three muscles differently. Similarly, drug administration by single injection results in a rapid rate of ChE inhibition with maximum ChE depression approximately 30 minutes postinjection while a minipump inhibits ChE more slowly with maximum ChE depression occurring 4-12 hours following the minipump implant. Thus, the further possibility was present that the mode of drug administration might result in differences in the severity of morphological alteration. These data indicate that acute and subacute doses of pyridostigmine which produce an approximate ChE depression of 60-70% result in morphological alterations to diaphragm, soleus and EDL NMJs which are variable within each muscle analyzed. The diaphragm appeared to reflect greater pre- and postsynaptic alterations in at least some NMJs. However, the differences were not consistent enough or significant to allow a subjective determination that the diaphragm is more sensitive to pyridostigmine exposure. Quantitation of the alterations would be necessary to positively determine if significant differences exist in the drug sensitivity levels of the three muscles. Thus, at these dose levels, drug induced alterations did not appear to be selectively associated with a specific muscle type or mode of drug administration with analysis of animals subjected to short-term exposure. The possibility exists that selectivity might be observed at different dose levels.

With increased periods of analysis following single injection or 14 osmotic minipump exposure, recovery was evident in all three muscles. While evidence is somewhat subjective, it appears that the nuclei in NMJs from animals in the 14 day subacute exposure group possessed aberrant nuclei with extremely irregular margins after 60 days of recovery than did the NMJs from animals in the acute exposure group. The significance of this observation is not currently known. However, nuclei of this type are normally associated with cells very high metabolic levels.

The following conclusions were drawn from the experiments performed in Contract Year 2.

1. At a ChE depression of 60 - 70 %, no discernable difference in the severity of NMJ damage is apparent between pyridostigmine exposure by single injection or osmotic minipump.
2. Ultrastructural alterations to NMJs of diaphragm, soleus and EDL were similar in extent and nature following short term exposure to pyridostigmine. Thus, at a ChE depression of 60 - 70 %, pyridostigmine exposure does not

appear to selectively affect one muscle fiber type more severely than another.

3. Diaphragm, soleus and EDL NMJs all reflect evidence of recovery from acute and subacute exposure to pyridostigmine following 60 day drug-free period. Current data indicate that the soleus may require a more extensive recovery period than the diaphragm and EDL.

RECOMMENDATIONS

Pyridostigmine-induced alterations occur at the NMJs of three muscle types studied, and thus established the toxic effects of the drug on fast twitch (EDL), slow twitch (soleus) and mixed (diaphragm) muscles. However, contract work completed in Years 1 and 2 has provided strong evidence that the damage which follows pyridostigmine exposure is reversible in all three muscles. Furthermore, recovery processes were evidenced even after moderately high drug exposures which induced whole blood ChE depressions of 70-90%. Since the potential for recovery from pyridostigmine-induced damage appears to be substantial, further evaluation of the drug as a potential prophylactic agent against intoxication by organophosphate agents is strongly encouraged. Specifically, the following recommendations have been formulated.

1. Extended recovery studies (similar to those described herein) particularly of animals exposed to moderately low levels of pyridostigmine (20-30% ChE depression) for 14-21 days should be performed to determine a time course for complete recovery. These data would allow more precise estimates of anticipated recovery rates in humans.

2. A series of experiments should be designed to assess the mechanism of damage at the NMJ. Specifically, the pre- and postsynaptic movement and accumulation of ions involved in neuromuscular transmission should be evaluated. A more thorough understanding of the molecular mechanism of the effects of pyridostigmine would allow more judicious selection of dose and duration of exposure in military personnel.

3. A series of experiments should be designed to assess the acute and subacute effects of pyridostigmine on exercised animals or on in vitro stimulated muscles. A study of this nature could reveal functional and/or behavioral alterations not manifest in rested animals. Such data would provide a more substantial base for predicting pyridostigmine

effects on military personnel functioning under conditions of stress.

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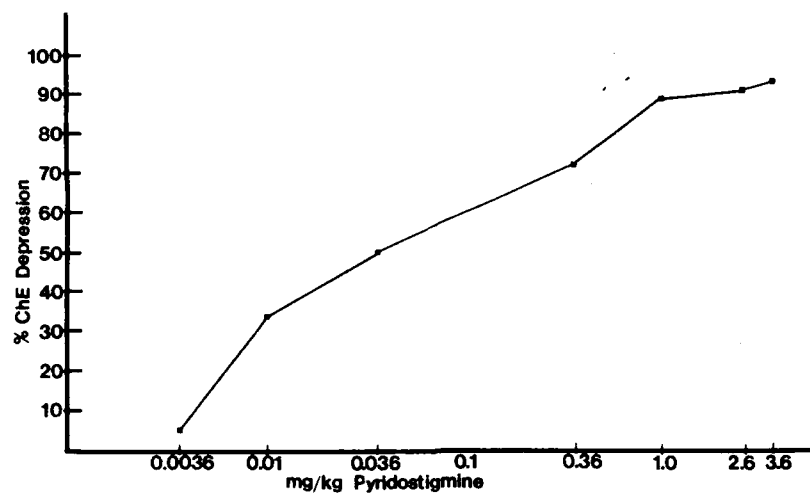
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FIGURE LEGENDS

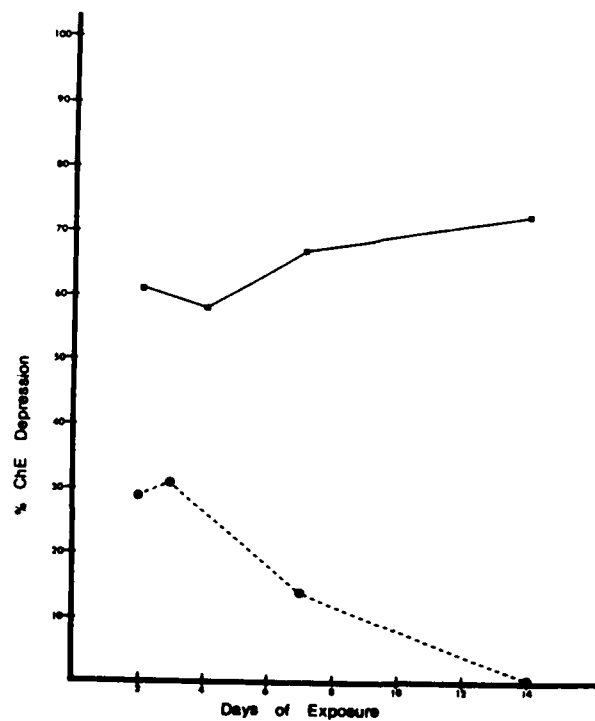
Graph 1. Acute dose responses of whole blood ChE depression (% inhibition) induced by single subcutaneous injections of pyridostigmine bromide ranging from 0.0036 mg/kg (0.001 LD50) to 3.6 mg/kg (1 LD50). The percent depression was determined by calculating the ^{14}C activity in the pre-injection and post-injection (30 min) whole blood samples from each animal. The mean pre- and post-injection activity was determined for each experimental group and these values were used to calculate the percent ChE depression which was plotted against dose on a semi-log scale. Minimum n = 3-4 animals per dose.

Graph 2. Cholinesterase depression (% inhibition) resulting from continuous subcutaneous infusion of pyridostigmine bromide via 2 ml osmotic minipump. The percent depression was determined as for acute dose response studies. The ^{14}C activity was determined for blood samples from each animal prior to and at intervals following the implant of a drug-containing osmotic minipump. The mean pre-exposure values were compared to mean post-exposure values to determine the percent ChE depression in each experimental group at each time point. Inhibition is shown for a high dose (solid line) of 10 mg/ml (total exposure at day 14 = 20 mg) and a low dose (dotted line) of 1.5 mg/ml (total exposure at day 14 = 3 mg). Minimum n = 4 animals per data point.

G1



G2



Figures 1 and 2. EOL NMJs from acute (1) and subacute (2) control animals treated with Mestinon -equivalent buffer. The relationship of the nerve terminal (n) Schwann cell (s) and postsynaptic junctional folds (j) appears unaffected by exposure to the buffer. The nerve terminal is evenly apposed the the junctional folds and separated by the typical 50 nm primary cleft which contains the basal lamina. Note that the overlying Schwann cell processes do not enter the primary cleft. Postsynaptically, the junctional folds and sarcomeres reflect no signs of alteration.



Figure 3. A diaphragm NMJ from an animal exposed to 0.36 mg/kg pyridostigmine for 30 min. reveals a partially withdrawn nerve terminal (asterisk) which possesses densely packed synaptic vesicles. Schwann cell processes are present in the synaptic cleft. Postsynaptic mitochondria (arrows) have abnormal rarefied regions of matrix.

Figure 4. An exposure of 0.36 mg/kg pyridostigmine has altered pre- and postsynaptic mitochondria of this soleus NMJ. Note particularly the postsynaptic mitochondria which contain densely packed layers of membrane (arrows).

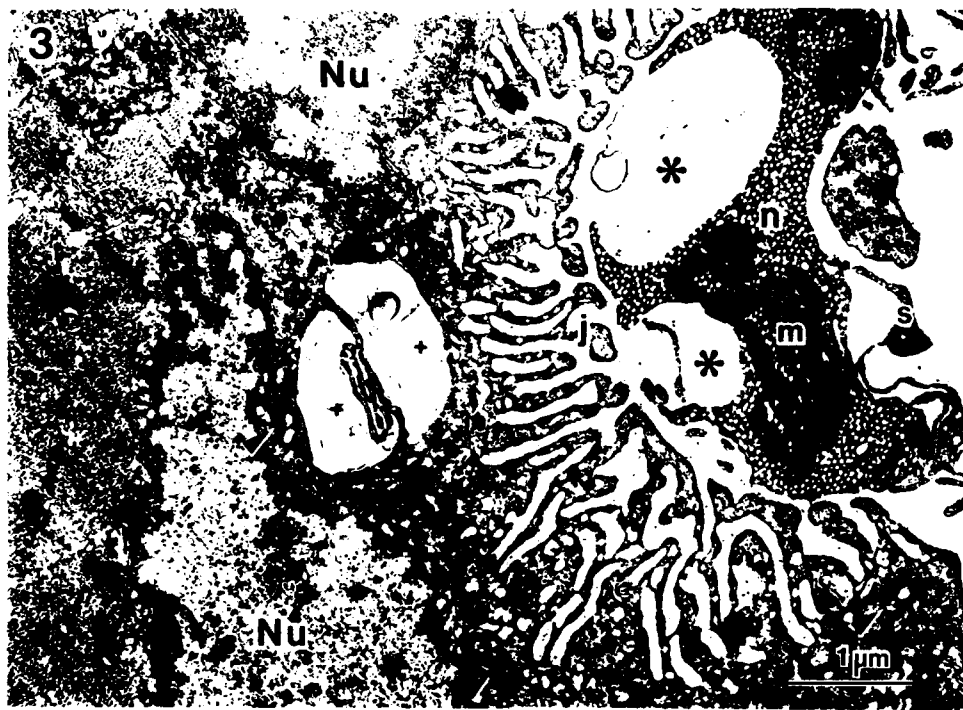
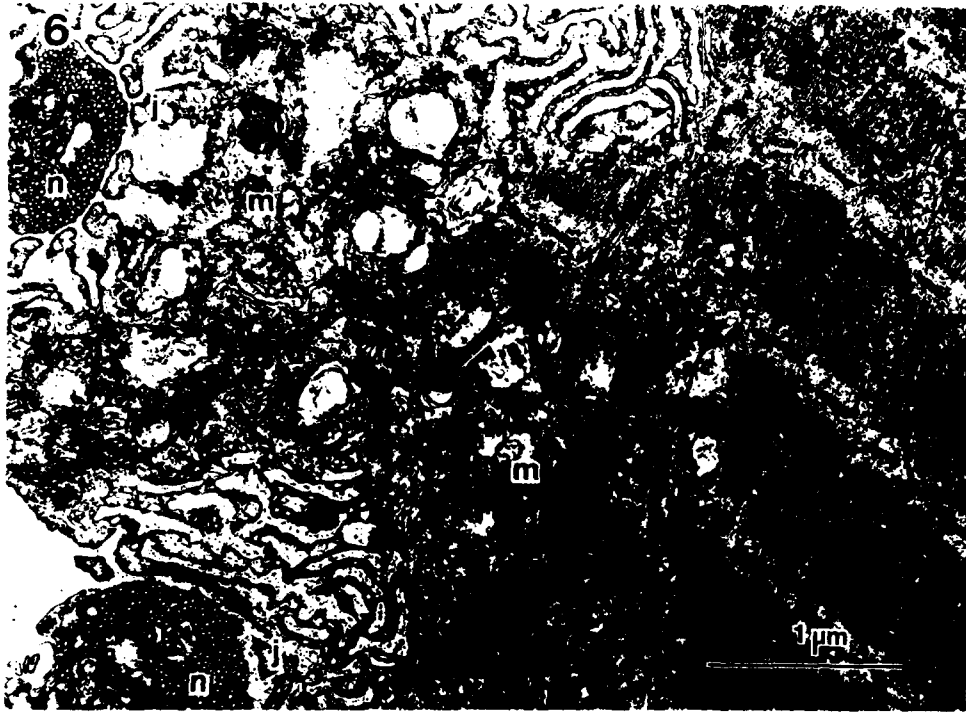


Figure 5. An EDL NMJ exposed to 0.36 mg/kg pyridostigmine has altered pre- and postsynaptic mitochondria. Some postsynaptic mitochondria contain densely packed layers of membrane (arrows). Other features of this NMJ appear unaffected by the drug exposure.



Figures 6 and 7. Diaphragm NMJs from two animals that received subacute exposure of drug via osmotic minipump reflect the variability of damage that can be observed. Pre- and postsynaptic mitochondria show signs of damage in both NMJs. However, while the sarcomeres in Fig. 6 have retained a normal pattern of organization, those in Fig. 7 have smeared Z bands (arrows) and disarrayed myofibrils.



Figures 8 and 9. Soleus (8) and EDL (9) NMJs which received a total subacute exposure of 2.8 mg of pyridostigmine (2 day) have moderate presynaptic damage. Note the regions of partial withdrawal of nerve terminal from the junctional fold crests (asterisks). In some of these areas Schwann cell processes are present in the synaptic cleft. Only the EDL NMJ (9) reflects drug-induced damage to the myofibrillar structure (arrows).



Figure 10 and 11. These diaphragm NMJs are from rats exposed to a single injection of 1 mg/kg of pyridostigmine. The NMJ in Fig. 10 was taken 30 minutes postinjection while the NMJ in Fig. 11 was taken 60 days postinjection. The 30 minute exposure has induced regional separation of the nerve terminal from the junctional fold crests (asterisk) and large rarefied areas in the subjunctional mitochondria. In addition, some Z bands and myofibrils have lost the precise organization normally present. 60 days following exposure, mitochondrial and sarcomere damage are no longer present. However, regional separation (*) of pre- and postsynaptic elements by Schwann cell (s) intervention in the cleft is common in this NMJ.

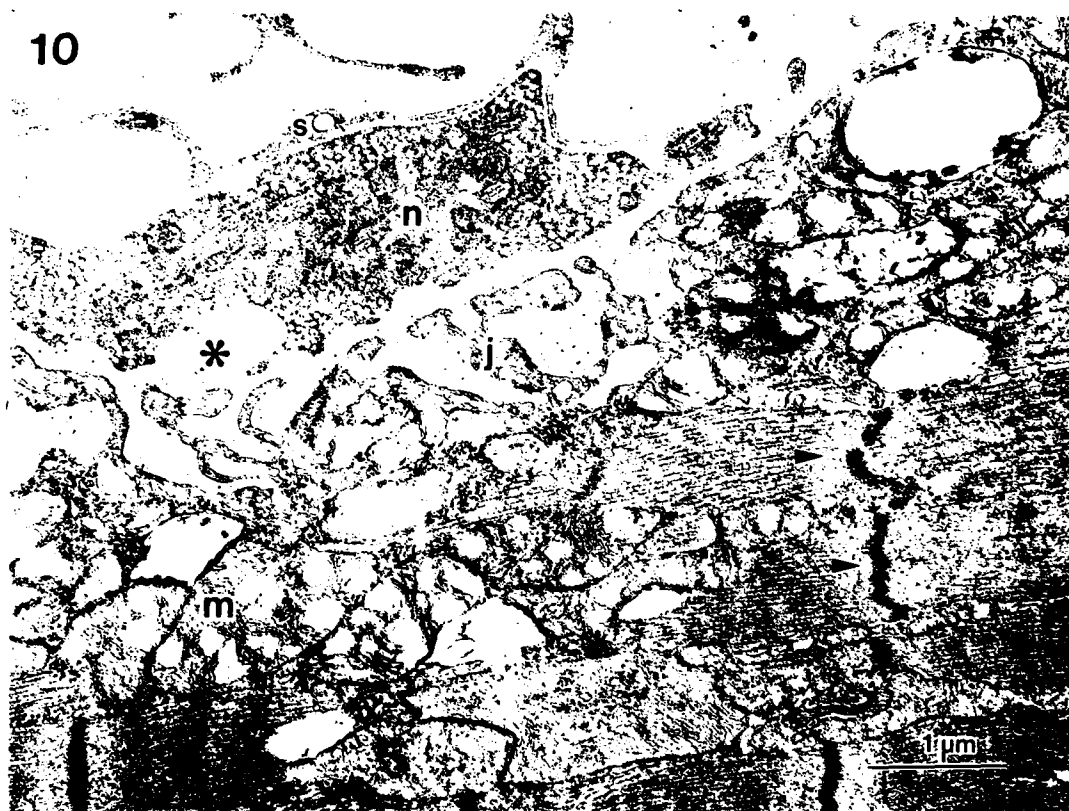
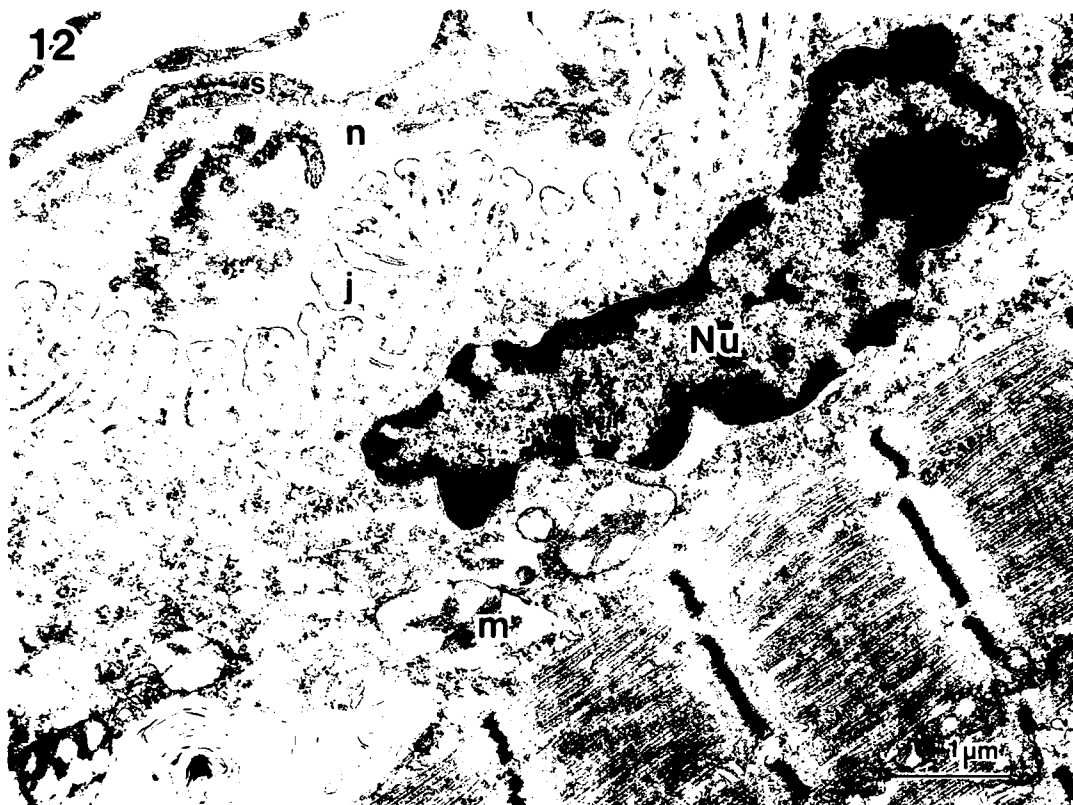


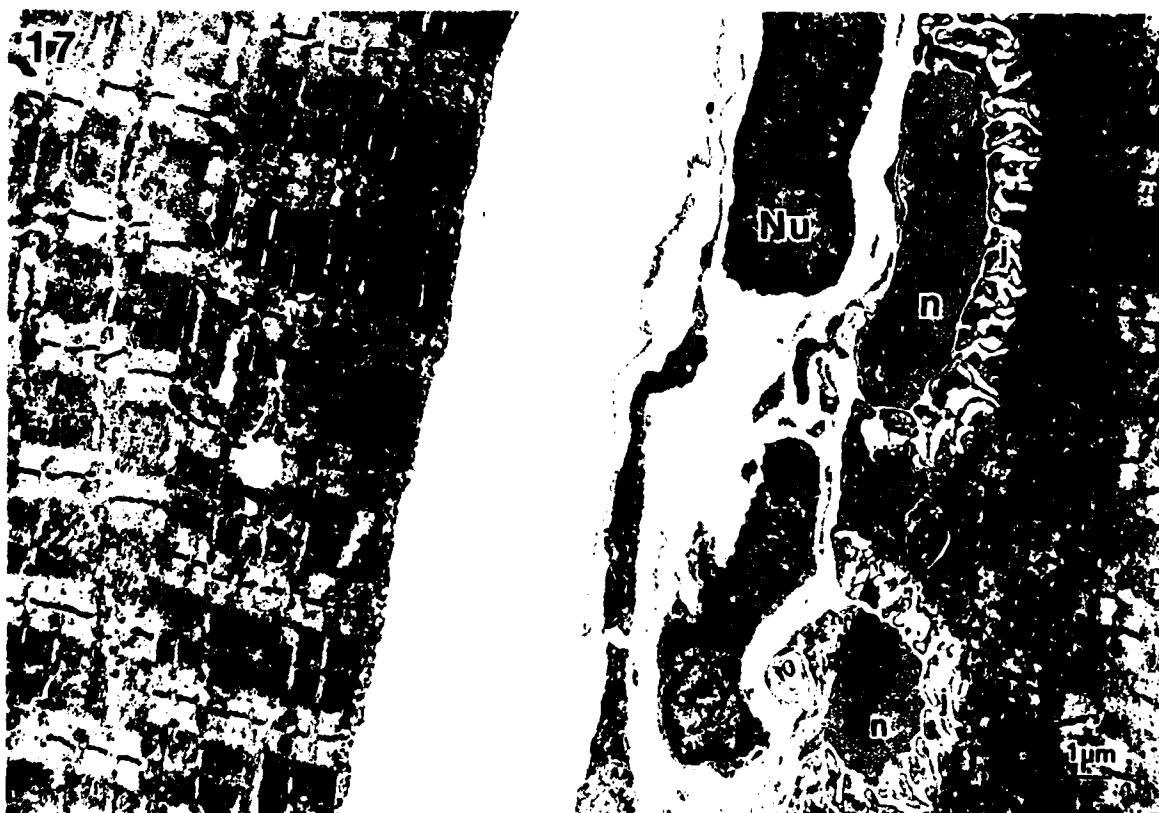
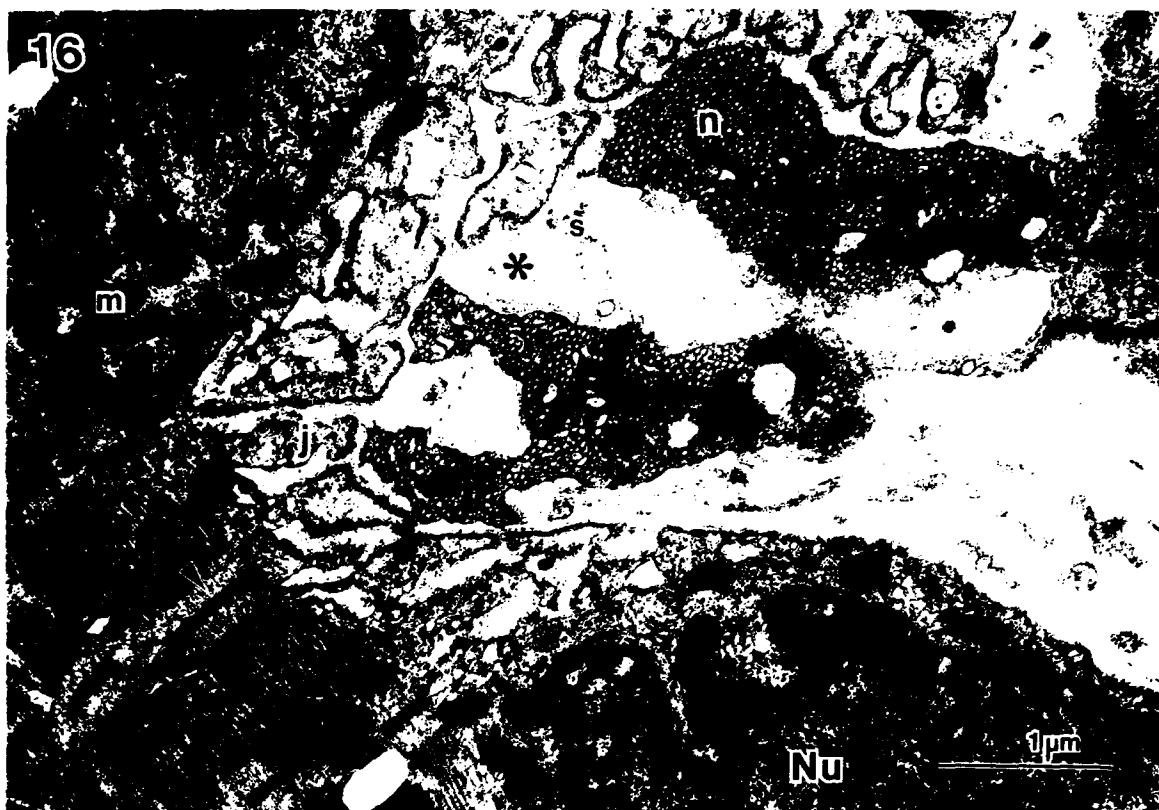
Figure 12 and 13. NMJs from soleus muscles exposed to 1 mg/kg. The muscles were collected at 30 minutes (12) and 60 days (13) postinjection. The nerve terminal shows no marked signs of alteration. However, a small process of Schwann cell is present in the synaptic cleft (arrow). With this short period of exposure, the postsynaptic mitochondria (m) are obviously affected. A large area containing multi-layered membrane fragments is apparent. Even after a recovery period of 60 days, damage persists in the second NMJ. Some presynaptic mitochondrial damage is evident. Postsynaptically, multilamellar bodies (arrows) persist.



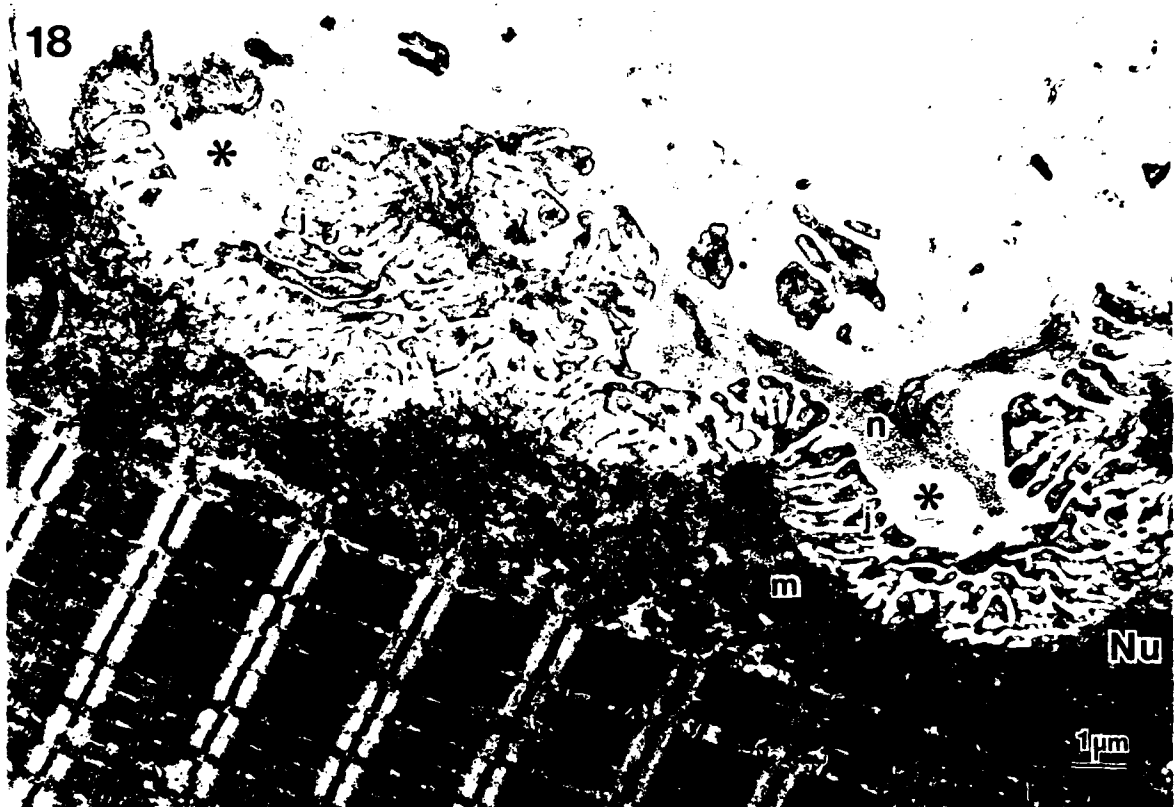
Figures 14 and 15. These two EDL NMJs illustrate the variation in the extent of damage in different muscle fibers 30 minutes after a single injection of 1 mg/kg of pyridostigmine. The NMJ in Fig. 14 possesses moderate alterations in presynaptic mitochondria and has apparent residual bodies present postsynaptically. The NMJ in Fig. 15 reflects considerable ongoing changes in ultrastructure. Portions of nerve terminal are surrounded by Schwann cell in the right hand area of the micrograph reflecting probable partial denervation processes. Postsynaptically, the mitochondria are swollen and the myofibrillar components lack typical organization.



Figures 16 and 17. Diaphragm NMJs exposed by osmotic minipump to 20 mg of pyridostigmine over a 14 day period illustrate the potential of repair processes. The NMJ in Fig. 16 reflects damage present at 14 days exposure. The nerve terminal shows large regions of separation from postsynaptic folds (*) with concomitant Schwann cell (s) intervention in the synaptic cleft. Postsynaptically, the sarcomeres are much shorter than rest length sarcomeres from nonjunctional areas (not shown) of the same muscle fiber. The NMJ in Fig. 17 received the same drug exposure but remained drug-free for an additional 60 days. No obvious signs of damage are present.



Figures 18 and 19. NMJs from two soleus fibers which received a 14 day subacute exposure of 20 mg of pyridostigmine reflect considerable presynaptic damage. The nerve terminal branches in Fig. 18 are widely separated from the junctional fold crests in several places (*). In Fig. 19 pre- and postsynaptic separation is due to the presence of Schwann cell processes in the synaptic cleft. Note the irregular margins of the postsynaptic nucleus.



Figures 20 and 21. These EDL NMJs reflect the effects of a 14 day exposure to 20 mg of pyridostigmine via osmotic minipump. On the 14th day, the NMJ in Fig. 20 possesses moderately altered pre- and postsynaptic mitochondria. One region of nerve terminal is notably separated (*) from the postsynaptic folds. Following 60 days of recovery from the drug exposure, the NMJ in Fig. 21 possesses morphology similar to that seen in control NMJs. However, the nucleus has an extremely irregular margin and is associated with an abnormal number of Golgi.

